PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 9454
Varadi *et al.*	Examiner: Rosanne Kosson
Application No.: 10/816,099	Technology Center/Art Unit: 1653
Filed: March 31, 2004	DECLARATION UNDER 37 C.F.R. § 1.132 BY DR. PETER TURECEK
For: KITS FOR MEASURING THROMBIN GENERATION	
Customer No.: 44183	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Peter Turecek, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

2. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

3. I am a named inventor on the above-referenced patent application. I have previously submitted two Declarations under 37 C.F.R. § 1.132 in support of the patentability of this application over various references cited in an obviousness rejection. However, the Examiner contends that the previous Declarations are insufficient to overcome the rejection (see, the Office Action dated May 1, 2008). My understanding of the reasons is as follows: First, the Examiner alleges that a proper comparison was not provided in the previous Declarations

because no data were presented that compared the solubility of a lyophilized mixture that contains both the substrate and $CaCl_2$ with the solubility of a lyophilized substrate where the substrate is lyophilized separately without the $CaCl_2$. Next, the Examiner also alleges that the previous Declarations were insufficient because the data were obtained using higher concentrations of fluorescent thrombin substrate and $CaCl_2$ solutions than the final concentration in the cited reference, Váradi *et al.* Last, the Examiner also contends that the data previously submitted suggested that the solubility in those experiments was also influenced by the container, *i.e.*, whether or not the substrate/$CaCl_2$ is prepared in glass vials or plastic wells. This Declaration is provided to further address these issues.

Preparation of Substrate Solutions

4. In the experiments presented here, different substrate samples were prepared as indicated on the attached flow sheet (Exhibit 1) to provide four samples that were directly compared in terms of solubility. For all of the experiments, the solutions were prepared and analyzed in glass sample containers to directly compare the solubility of the samples. Solubility was assessed visually (see, the photographs provided in Exhibit 2 for the various re-solubilization experiments described below).

5. Initially, 250 mg of the fluorescent-labeled thrombin substrate Z-Gly-Gly-Arg-AMC.HCL powder was weighed in a 100 ml flask and dissolved by stirring with a magnetic stirrer in 74 ml of 25 mM HEPES, 175 mM NaCl pH 7.35 buffer, containing 10% DMSO. After the powder was fully dissolved, 6 ml water was added to bring the volume of the substrate solution to 80 ml (in the experiments in the previous declarations, 6 ml of 1M $CaCl_2$ was added instead of the water). The solution remained clear upon the addition of the water. This solution has a substrate concentration of 5 mM in 24 mM HEPES, 175 mM NaCl – 9.25% DMSO buffer. The solution was further processed as shown in the flow chart in Exhibit 1.

6. The 80-ml substrate solution containing substrate was divided into parts and aliquoted as shown in the flow chart. This procedure resulted in four different substrate samples, two of them lyophilized without and two of them lyophilized after the addition of $CaCl_2$. The concentrations of substrates in the four samples are as follows:

Sample 1: The concentration of substrate in sample 1 (prior to lyophilization) was 5 mM with 10% DMSO--no $CaCl_2$ is present in the lyophilized sample;

Sample 2: The concentration of substrate in sample 2 (prior to lyohpilization) was 2.5 mM, 5% DMSO--no $CaCl_2$ is present in the lyophilized sample;

Sample 3: The concentration of substrate in sample 3 (prior to lyophilization) was 1 mM. The lyophilized sample also contains 15 mM $CaCl_2$ and 2% DMSO; and

Sample 4: The concentrations of substrate, $CaCl_2$, and DMSO are the same as Sample 3; the concentrations of the buffer components are different.

Sample 4 had the same composition as described in Example 6 (starting at paragraph 50) in the patent application. This is the minimum concentration of the substrate and $CaCl_2$ used here. During the preparation of the calcium-containing samples no precipitation was visible upon the addition of $CaCl_2$ dissolved either in water (Sample 3) or in buffer (Sample 4) prior to lyophilization. The solutions containing the thrombin substrate without $CaCl_2$ for samples 1 and 2 were also clear prior to lyophilization.

Reconstitution of the Lyophilized substrate Samples

7. The vials were reconstituted and prepared to reflect a "ready to use" solution, containing 1 mM fluorescent substrate and 15 mM $CaCl_2$ in different experiments, as described below. Each resolubilization procedure was performed at least in duplicate.

8. *Experiment 1*

The lyophilized powder in the vials of Sample 1 (5 mM Substrate prepared in 25 mM HEPES, 175 mM NaCl + 10% DMSO – Buffer) strongly adhered to the wall of the vials. After addition of 1 ml water for injection (WFI) the substrate was barely soluble, the powder continued to stick on the glass wall of the vial. To obtain a clear solution we had to vigorously mix the solution with a vortex and warm it to 37°C.

After the substrate was dissolved in the 1 ml of water, the required amount of $CaCl_2$ to reach a final concentration of 15 mM $CaCl_2$ was added in three different ways:

a. 4 ml of 19 mM $CaCl_2$ solution (in water) was added;

b. 4 ml of 19 mM $CaCl_2$ in 25 mM HEPES, 175 mM NaCl pH 7.35 buffer was added; or

c. First 1 ml of 25 mM HEPES, 175 mM NaCl pH 7.35 buffer was added, followed by 3 ml of 25 mM $CaCl_2$.

No opalescence or precipitation was observed upon the addition of $CaCl_2$ to the dissolved substrate.

9. *Experiment 1a*

As noted above, the lyophilized powder in vials of Sample 1 (5 mM Substrate prepared in 25 mM HEPES, 175 mM NaCl + 10% DMSO – Buffer) strongly adhered to the wall of the vials and was present on the glass wall. In order to compare the solubilizing protocol in Experiment 1 to a protocol using one step to bring the solution to the desired concentrations, 5 mls of 15 mM $CaCl_2$ were added to the lyophilized powder to attempt to re-solubilize it. As in Experiment 1 using 1 ml of water, the substrate was barely soluble, the powder was sticking to the glass wall of the vial. To obtain a clear solution, we again had to vigorously mix the solution using a vortex and warm it to 37°C.

10. *Experiment 2*

The lyophilized powder in the vials of Sample 2 (2.5 mM Substrate prepared in 25 mM HEPES, 175 mM NaCl -5 % DMSO buffer) also strongly adhered to the wall of the vials. After addition of 1 ml water for injection (WFI), the substrate was barely soluble, the powder was sticking to the glass wall of the vial. To obtain a clear solution, it was also necessary to vortex it vigorously and warm the solution to 37°C.

After the substrate was dissolved, the amount of $CaCl_2$ to bring the final concentration to 15 mM was added in two buffer media:

a. 1.5 ml of 25 mM $CaCl_2$ solution (in water) was added; or

b. 1.5 ml of 25 mM $CaCl_2$ solution 25 mM HEPES, 175mM NaCl pH 7.35 buffer was added.

No opalescence or precipitation was observed upon the addition of $CaCl_2$ to the dissolved substrate in either case.

11. *Experiment 3*

The lyophilized powder in the vials of Sample 3 (1 mM Substrate; 15 mM $CaCl_2$ in 10 mM HEPES; 70 mM NaCl; -2% DMSO buffer) formed a thin film that was visible at ~ 2/3 of the glass wall. Because this sample had a lower buffer concentration, two different dissolving strategies were applied:

a. Reconstitution with 1 ml of water for injection (WFI). In contrast to the Experiments 1 and 2, after addition of 1 ml of water the substrate easily dissolved after a short vortexing.

b. Reconstitution with 0.2 ml of water for injection (WFI), followed by the addition of 0.8 ml of 25 mM HEPES, 170mM NaCl pH 7.35 buffer. The addition of the small volume of WFI resulted in an opalescent solution, which readily became clear upon dilution to the original lyophilization volume (1 ml) with the buffer.

12. *Experiment 4*

The composition of the lyophilized powder in the vials of Sample 4 (1 mM Substrate; 15 mM $CaCl_2$ in 25 mM HEPES; 175 mM NaCl; -2% DMSO buffer) was similar to those described in Example 6 of the specification.

Upon reconstitution with 1 ml of water for injection (WFI), the lyophilized powder dissolved immediately, resulting in a "ready to use" reagent for TGA.

Conclusion

13. The present invention relates to the discovery of a procedure where a non-water-soluble fluorescence substrate can be converted to a water-soluble, "ready to use" reagent. In various examples, including Example 6 of the specification, the procedure includes a step in which $CaCl_2$ is added to a concentrated substrate solution, resulting in a precipitate formation, which can only be dissolved with difficulties. After re-solubilization and dilution to the desired concentrations, the substrate-$CaCl_2$ solution is lyophilized as a mixture, and, surprisingly, the resulting powder becomes very easy to dissolve in water.

14. In the experimental series presented in this Declaration, the substrate was dissolved and diluted (see, initial preparation procedure) before the addition of $CaCl_2$. In one set of samples (samples 1 and 2), the substrate (clear solution) was lyophilized before the addition of $CaCl_2$, such that the solubility of lyophilized substrate without the $CaCl_2$ could be compared to the solubility of the lyophilized substrate mixture that contains $CaCl_2$. When the substrate was lyophilized without $CaCl_2$ present in the mixture, the lyophilized powder was largely insoluble (it required long and vigorous shaking, mixing and warming to dissolve) (see, reconstitution experiments 1, 1a, and 2). As a consequence, this kind of product would not be regarded as a "ready to use " reagent.

15. In the other experiments (samples 3 and 4) in which the $CaCl_2$ was added to the diluted, dissolved substrate (no precipitate was visible) and then the substrate and $CaCl_2$ were lyophilized together, the lyophilized mixture could easily be reconstituted with water for injection (WFI).

16. The experiments presented in this Declaration thus provide additional evidence that it is necessary to lyophilize the substrate together with the $CaCl_2$ to produce an easy water-soluble "ready to use" substrate reagent. Based on my experience in this field working with fluorescent-labeled thrombin substrates, this is a new, surprising effect to convert a non-water-soluble product to a water-soluble product.

17. The Declarant has nothing further to say.

Date: Nov. 7. 2008

By: _____
Peter Turecek, Ph.D.